

Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

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LONG-TERM GOALS

Quantifying physiological indicators of stress in wild marine mammals and the interrelationships between different stress markers can be used to estimate the impact of anthropogenic stressors on marine mammal populations. The United States Navy, as part of its environmental stewardship, can utilize stress markers to assess the acute and chronic impacts that its actions might have on marine mammals. This approach would permit better mitigation of potential impacts and ensure that Navy activities do not come at a deleterious cost to marine mammal populations.

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OBJECTIVES

The objectives of this effort are to: 1) determine the variation in corticosteroid hormones, thyroid hormones, and catecholamines within a dolphin population relative to seasonality, time of day, gender, age and reproductive state; 2) assess relationships between serum corticosteroid levels and levels found in other matrices (i.e. biological samples), including feces and blubber; 3) and to perform adrenocorticotropic hormone (ACTH) and thyrotropin-releasing hormone (TRH) challenges to characterize the activation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axes across multiple matrices, respectively.

APPROACH

Task 1 – Seasonal variations in hormones across multiple matrices

Regular sampling from different matrices (e.g. blubber, blood, feces) will be collected from the U.S. Navy Marine Mammal Program (MMP) dolphin population over the course of a year. Subject dolphins will be split into categories based upon age: 5-15 years, 16-25 years, and 26-35 years. Each animal will be sampled bi-weekly throughout the year for blood and feces. A subset of animals will be selected for blubber biopsies, which will be conducted every month.

Blood samples will be collected from dolphins through their voluntary participation. Blood collections will be made from the ventral fluke from the arteriovenous plexus and collections will be made between 0700-1000. Fecal samples will be collected by use of a suction catheter inserted into the anus of the dolphin. Fecal samples will be collected through voluntary cooperation and will be performed the day after the blood collection. Bimonthly blubber biopsies will be collected with a 16g or 18g tissue biopsy needle and the condition and healing of the animal biopsied will be monitored daily following the procedure.

Serum samples will be processed for adrenocorticosteroids, catecholamines, and thyroid hormones via radioimmunoassay (RIA). Radioimmunoassay methods have previously been validated for cortisol and aldosterone in this species (Houser et al., 2011). Parallel processing of serum catecholamines will be performed via high-performance liquid chromatography (HPLC) to assess variability in the measurement methods available for these hormones..

Metabolites of cortisol, aldosterone and thyroid hormone will be extracted from fecal samples and measured via RIA using established techniques (Wasser et al., 2010). Thyroid measures will focus on T3 because I¹³¹ ingestion studies showed that thyroid hormone was excreted in feces almost entirely as T3 with very little T4 in two domestic dogs, and similarly only immunoreactive T3 was primarily found in killer whale feces with lesser amounts of T4 (Wasser et al., 2010).

A multi-step biphasic organic solvent extraction will be used to isolate the corticosteroids from the blubber tissue (Kellar et al., 2009). The hormones will be measured using a commercially available enzyme immunoassay (EIA) and parallel processing via HPLC will be used to verify method performance.

Task 2 – Diurnal variation in hormone production

Hormones will be assessed for diurnal variation during the second year of the study with the goal of assessing changes that occur between dawn and dusk. Ten dolphins will be selected for repeat testing throughout the year. Blood samples will be collected from the dolphins at monthly intervals via

voluntary venipuncture of the arteriovenous plexus on the ventral fluke. Triplicate blood samples will be collected on the day of sampling. The initial samples will be collected first thing in the morning (~0700), the second at noon, and the third in the late afternoon (~1700). Blood samples will be processed via RIA and HPLC as described under Task 1. Similar analyses will be conducted on serially collected scat of these 10 individuals over the same 24 hr period and a second 24 hr period one week later when not being sampled for blood.

Task 3 – Adrenocortical sensitivity

Adrenocortical sensitivity and the relationship between activation of the HPA axis and reflection of this activation in serum and other matrices will be determined. The information from this assessment will allow researchers to better understand the temporal and quantitative relationship between hormones measured in matrices likely to be collected from wild animals, namely feces and blubber, and that circulating in the blood stream.

Three animals will be selected for determining the appropriate dose of ACTH required to sufficiently elevate the corticosteroids in serum and other matrices. ACTH slow-release gel will be intramuscularly implanted to permit time-controlled and sustained release of ACTH. Implantation of the gel will be performed by MMP or NMMF veterinarians and the animal will be monitored for several hours following the injection. Repeat blood samples will be taken over a course of several days to determine the relationship between the time course of serum corticostroid increase and the ACTH dose administered. Based upon results of the pilot study, a schedule will be determined for the collection of samples from other matrices (feces and blubber) that will be tested during the second and third years of the study. Five dolphins will be selected during for ACTH challenges following conclusion of the pilot. For each of the dolphins, blood, feces and blubber samples will be collected according to the sampling schedule determined during the first year of the study.

A pilot study will also be conducted in which a dolphin is fed fish containing cortisol pellets. For the first pilot study, 10 mg of cortisol will be fed to the dolphin in fish at six hour intervals over multiple days to attempt to raise and maintain the serum cortisol levels. Voluntary blood samples will be collected across multiple days and different time frames to determine if serum cortisol levels are elevated and sustained. Based on the results, the pilot will either be repeated with an increase in the cortisol dosage or frequency of delivery, as determined in consultation with the attending veterinarian. Provided the procedure adequately raises cortisol levels, the process will be repeated with five bottlenose dolphins and will be coupled to blubber biopsies so cortisol deposition in the blubber can be assessed.

Task 4 – Thyroid challenges

Thyroid hormones (thyroxin, T4 and triiodothyronine, T3) are released from the thyroid gland and are responsible for regulating the metabolism of an animal and affect the activity of other stress hormones via permissiveness. Thyroxin is the more abundant of the two thyroid hormones in circulation and the metabolic parent hormone. However, the bioactive form is largely T3, which is roughly eight times more potent than T4 (Tomasi 1991). Thyroid hormone production is known to be affected by stress, which can lead to conditions of both hypo- and hyperthyroidism. Persistent elevated or diminished levels of these hormones are known to lead to pathophysiological conditions that can ultimately impact important life history functions.

Thyroid hormones are produced in response to the presence of thyroid stimulating hormone (TSH), which is a peptide hormone produced in the anterior pituitary gland. Thyroid stimulating hormone is

itself produced in response to the action of thyrotropin-releasing hormone (TRH), which is produced in the hypothalamus. Assessing the responsiveness of the production pathway to acute elevations of TRH, i.e. a hormone challenge, is one means by which pathway responsiveness and activity of TRH can be quantified at different levels of the synthesis pathway.

Three dolphins will be given an exploratory TRH challenge to determine the optimal dosing and sampling schedule. A pre-test blood draw will be collected from the dolphin while it is in its enclosure. The dolphin will then be removed from the water to a location on the pier that is deemed suitable for the procedure by the attending veterinarian. A bolus injection of TRH will be intravenously administered via the venous plexus of the fluke, or an alternative route as deemed necessary by the attending veterinarian. Blood samples will then be collected every 15 minutes for a period of 4 hours. Following completion of the sampling period, the dolphin will be returned to its enclosure. The dolphin will be monitored following the procedure for a period of time to be determined by the attending veterinarian. Dosages of TRH will be adjusted for the second and third animal in the pilot study following analysis of the blood samples collected with the first challenge. Following the pilot study, eight individuals will be submitted to TRH challenges. Baseline blood and fecal samples will be collected prior to the first injection and blood collections will be performed as described for the pilot studies (with minor modifications to the blood draw schedule as determined by the pilot study results). Fecal samples will be collected for 96 hrs following injection.

WORK COMPLETED

Task 1 – Seasonal variation in stress hormones

A group of 30 bottlenose dolphins was identified from within the MMP population that could provide voluntary biweekly blood and fecal samples over a period of a year. One of the animals was removed from the study after several months and was replaced by a comparably aged animal of the same gender. The following distribution of animals was obtained:

Age (yrs)	Male	Female
5-15	6	4
16-25	3	3
25+	7	7

Four of the dolphins were identified for monthly blubber biopsies to be collected on the same day of the blood collections.

A total of 735 blood collections were made out of a total of 778 possible draws (~94% success rate). A total of 638 matched fecal samples were collected such that 87% of the blood samples had matched fecal comparisons. Three of the female dolphins in the study became pregnant during the course of the study. Excepting the one dolphin that birthed, the pregnancies did not significantly interfere with sample collection.

Blubber biopsies were collected approximately 12-14 cm below the posterior insertion of the dorsal fins using a 16g biopsy needle. Two to three biopsies were taken each sample period to ensure that sufficient blubber was obtained for analysis. A total of 47 blubber biopsies were collected over the course of the study.

Sample collection on this task is complete. Approximately 75% of all blood samples have been analyzed. Approximately 83% of the blubber samples have been processed and all samples for HPLC analysis and fecal hormone analysis are complete.

Task 2 – Task 2 will be completed by the end of September 2013. To date, 99% of the scheduled blood samples have been collected. Samples have been archived at -80°C but have not yet been processed.

Task 3 – Five cortisol feeding trials were completed in FY13. Based on the results of pilot studies, the dose of cortisol was elevated to 60 mg every six hours over a period of five days. Daily voluntary blood and fecal samples were collected and blubber biopsies were taken prior to cortisol feeding (day 0) and on the third and fifth day of feeding. Samples have been archived but have not yet been completely processed. Preliminary results indicate that cortisol is sufficiently elevated by feeding that ACTH production drops precipitously, in accordance with expectation. Furthermore, the biological half-life of cortisol was calculated from the study to be ~109 minutes.

Four ACTH stimulation pilot studies have been completed. As a precautionary approach, ACTH dosages have been step-wise increased from 0.02-0.15 U/kg. Initial results have not demonstrated a sustained cortisol production in response to the ACTH administration.

RESULTS

Preliminary results indicate that dolphins at the MMP produce low levels of corticosteroids but do not suffer from adrenal exhaustion or insufficiency. In many instances, levels are sufficiently low that alternative means of processing are being employed to accurately assess circulating levels. The low circulating levels likely reflect the lack of predatory and foraging stressors as well as disease mitigation. Thus, the population is a good system from which to determine baseline hormonal variations. Significant differences in thyroid hormones by season and year are evident, which is an important factor for understanding samples from wild-caught populations. An incidental finding of megestrol acetate administration impeding cortisol production was found in the MMP population. Due to the widespread use of megestrol acetate, this incidental finding has significant ramifications for the welfare of dolphins under human care.

Determination of the biological half-life of cortisol permits cortisol levels observed in wild and captive animals to be placed in better context of collection methods (e.g. impact of handling) and provides insight on the kinetics of this corticosteroid. It is anticipated that the impact on other hormones resulting from elevated cortisol will be determined within the upcoming year.

IMPACT/APPLICATIONS

The ability to identify stress markers relative to monitoring the health of marine mammal populations is critical to understanding the impact of anthropogenic activities upon those populations. The baseline characterization of stress marker variation in dolphins as a function of seasonality, gender, age, and reproductive status is important to assessing measurements made in wild dolphins. Information on levels and dynamics of stress markers between different matrices will provide better estimates of the overall condition of marine mammals sampled in the wild from either blubber biopsies or fecal collections. In addition, an understanding of the function of the HPA and HPT axis will provide fundamental information on the stress response in these marine mammals, which may differ significantly from that of the terrestrial mammals from which most of our understanding is based. The

incidental finding of the impact of MegAce on the dolphin endocrine system has broad-scale implications for the welfare of dolphins under human care.

RELATED PROJECTS

Project: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins

PI: Pat Fair

This project looks at numerous markers of stress in a wild population of marine mammals and compares them to animals under managed care in order to quantify and qualify the impact of environmental stressors on wild dolphins. The dolphins under managed care are from the Georgia Aquarium and the Navy Marine Mammal Program. Ten of the dolphins used in Task 1 of the current study (PI – Houser) were used as the semi-domesticated comparison.

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PUBLICATIONS

Houser, DS, Champagne, CD, Crocker, DE, Kellar, NM, Cockrem, J, Romano, T, Booth, RK and Wasser, SK. Natural variation in stress hormones, comparisons across matrices, and impacts resulting from induced stress in the bottlenose dolphin. In: *Effects of Noise on Aquatic Life II*, Popper, A and Hawkins, A, eds. [In Press].